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Pneumocystis murina Infection and Cigarette Smoke Exposure Interact To Cause Increased Organism Burden, Development of Airspace Enlargement, and Pulmonary Inflammation in Mice[▽]

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Chronic obstructive pulmonary disease (COPD) is characterized by the presence of airflow obstruction and lung destruction with airspace enlargement. In addition to cigarette smoking, respiratory pathogens play a role in pathogenesis, but specific organisms are not always identified. Recent reports demonstrate associations between the detection of *Pneumocystis jirovecii* DNA in lung specimens or respiratory secretions and the presence of emphysema in COPD patients. Additionally, human immunodeficiency virus-infected individuals who smoke cigarettes develop early emphysema, but a role for *P. jirovecii* in pathogenesis remains speculative. We developed a new experimental model using immunocompetent mice to test the interaction of cigarette smoke exposure and environmentally acquired *Pneumocystis murina* infection in vivo. We hypothesized that cigarette smoke and *P. murina* would interact to cause increases in total lung capacity, airspace enlargement, and pulmonary inflammation. We found that exposure to cigarette smoke significantly increases the lung organism burden of *P. murina*. Pulmonary infection with *P. murina*, combined with cigarette smoke exposure, results in changes in pulmonary function and airspace enlargement characteristic of pulmonary emphysema. *P. murina* and cigarette smoke exposure interact to cause increased lung inflammatory cell accumulation. These findings establish a novel animal model system to explore the role of *Pneumocystis* species in the pathogenesis of COPD.

Chronic obstructive pulmonary disease (COPD) is characterized by the presence of airflow obstruction and lung destruction. The main risk factor for developing COPD is cigarette smoking; however, only about 20% of smokers develop lung disease. The mechanisms by which cigarette smoke leads to pathological changes in both airways and lung parenchyma are unclear. Pulmonary inflammation, localized to the airways, is a pathological feature in the lungs of COPD patients. A predominance of macrophages and $CD8^+$ T lymphocytes are found both in bronchial biopsies (43, 44) and in the lung parenchyma at sites of parenchymal destruction (29). These findings suggest that $CD8^+$ T lymphocytes may be responsible for damage to the lung, either by directly injuring neighboring cells, by secreting molecules that injure the lung, or by recruiting and activating additional inflammatory cells.

The stimuli responsible for triggering inflammation are not known, but infectious pathogens likely play a role in the initiation or perpetuation of pulmonary inflammation in COPD. Much of the work examining the role of infection in the course and pathogenesis of COPD has focused on bacterial colonization (45), but bacterial or viral colonization may be insufficient to explain pathogenesis. An intriguing alternate pathogen that has received recent attention is the fungus *Pneumocystis jirovecii*. Although it was previously thought to be an opportunistic pathogen that caused serious pneumonia only in severely immunocompromised patients, recent investigations substantiate the existence of carrier states and colonization. In patients with COPD, for example, *P. jirovecii* DNA is present in approximately 20% of lung specimens obtained at the time of transplantation or lung resection, and the incidence of *P. jirovecii* colonization increases with worsening severity of COPD (35). *P. jirovecii* is also frequently found in the sputa of patients with pulmonary disease, and it has been identified in the sputa of approximately 50% of patients with chronic bronchitis (10).

Whether repeated bouts of *P. jirovecii* colonization or carriage could contribute to lung damage has not been investigated. It has long been established that the incidence of emphysema is significantly increased in individuals with chronic human immunodeficiency virus (HIV) infection (7, 27). Furthermore, accelerated development of emphysema has been identified in HIV-infected individuals who smoke cigarettes (15, 16). In a cohort study, about 15% of HIV-infected individuals demonstrated emphysema (indicated by pulmonary function testing and high-resolution computed tomography), compared with 2% of individuals in a matched control population (15). More recently, data from the Veterans Aging Cohort demonstrate that HIV-infected individuals are 50 to 60% more likely to have COPD than HIV-negative controls and that HIV infection is an independent risk factor for COPD (14). Although the mechanisms underlying this accelerated emphysema are unknown, chronic or repeated bouts of *P. jirovecii* infection or colonization are plausible explanations.

Because of the inherent difficulties in studying the development of COPD in humans, improved animal models are re-

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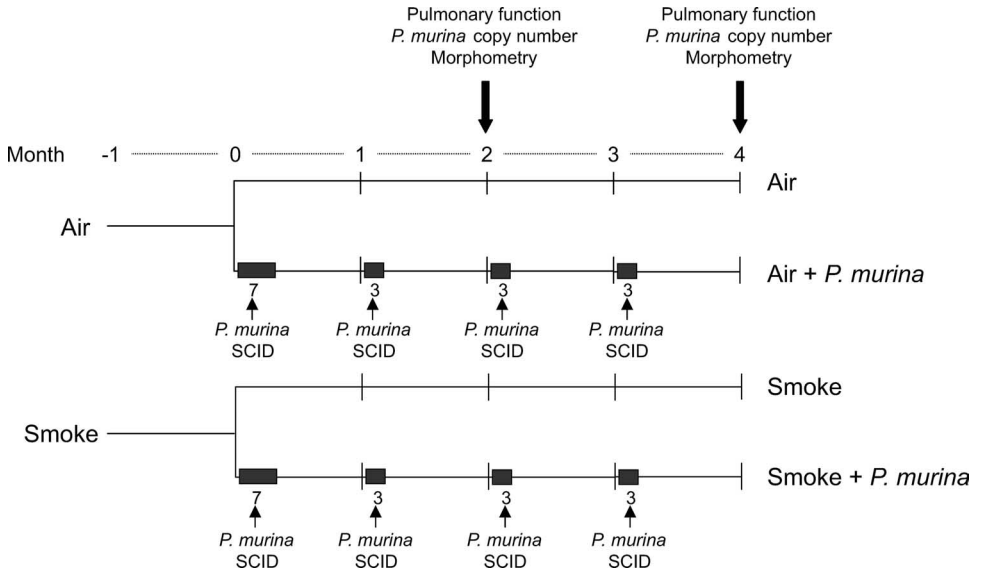

FIG. 1. Experimental design for experiments involving combined *P. murina* and smoke exposure. Groups of C57BL/6 mice were randomized to exposure to air or smoke and then were divided into groups exposed to a SCID mouse infected with *P. murina* or to a control group. Exposures by cohousing occurred for 7 or 3 days as indicated. Mice were assessed at 2- and 4-month time points after initial *P. murina* exposure. In parallel experiments, C57BL/6 mice were exposed to uninfected SCID mice for identical durations.

quired to test hypotheses about pathogenesis. Murine models of COPD using smoke exposure have recently received increased interest, because mice exposed to cigarette smoke develop emphysema, with some strains being more susceptible than others (21, 46). Because the inflammation observed after cigarette smoke exposure in mice is mild, this model lacks one of the key features of the human disease. One explanation of the failure of cigarette smoke alone to induce lung disease closely resembling human disease is that an additional insult is required (37). We reasoned that *P. jirovecii* would be a plausible pathogen to participate in both lung damage and inflammation.

Based on existing clinical and experimental data, we developed a new animal model that combines cigarette smoke exposure and environmentally acquired *Pneumocystis murina* infection in immunologically intact mice. We hypothesized that cigarette smoke and *P. murina* infection would interact to cause increases in total lung capacity (TLC) and functional residual capacity (FRC), increases in airspace size, and increases in lung inflammation, compared with either cigarette smoke or *P. murina* infection alone. In this model, we found that exposure to cigarette smoke exposure significantly increases the lung organism burden of *P. murina*. We also found that infection with *P. murina*, combined with cigarette smoke exposure, results in increases in lung capacity and in airspace enlargement characteristic of pulmonary emphysema. The combined insults caused pulmonary inflammation in excess of that observed with a single insult. These findings support a newly identified role for environmentally acquired *Pneumocystis* species in the pathogenesis of COPD and establish a novel, relevant animal model in which to test hypotheses about lung damage in the context of cigarette smoke exposure and *Pneumocystis* infection.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6 and C57BL/6 SCID mice were purchased from Jackson Laboratories and housed under specific-pathogen-free conditions in isolator cages. Autoclaved water and food were given ad libitum. The animal care committees at the University of Michigan and at the Ann Arbor Veterans Affairs Medical Center approved all protocols.

***P. murina* infection.** *P. murina* was propagated in SCID mice as previously described (48). Briefly, lungs harvested from SCID mice chronically infected with *P. murina* were removed and homogenized. Recovered *P. murina* organisms were inoculated intratracheally into subsequent generations of SCID mice. At 5 to 8 weeks after passage of *P. murina*, an infected SCID mouse was used as the source of infection for the cohousing experiments described below. After exposure to uninfected C57BL/6 mice, the SCID mice were removed from the cages and were euthanized to measure the intensity of *P. murina* infection by real-time PCR assay for *P. murina* rRNA (see below) in one lung. The remaining lung was processed for silver staining and histologic examination (5). To control for any environmental stress that could have been caused by exposure of C57BL/6 mice to SCID mice, additional experiments were performed using uninfected SCID mice, which in each case were found to be free of *P. murina* rRNA by PCR.

Cigarette smoke exposure. Immunologically intact C57BL/6 mice exposed to smoke from 2 cigarettes per day for 6 days a week tolerate exposure for at least 1 year and develop pathological changes of emphysema by 6 months of exposure (21, 46). We modified this method to use an environmental smoking chamber to accommodate larger numbers of mice. Cigarette smoke (University of Kentucky Tobacco Health Research Institute standardized cigarettes, lot 1R3F) was diluted to a 4% mixture (by volume) with dry compressed air. This gas mixture was passed into airtight exposure chambers containing cages of mice. To avoid possible cross-contamination with organisms, separate chambers were used for mice exposed to smoke alone and for mice exposed to smoke plus *P. murina*. Each exposure lasted for 4 h, 5 days per week. Mice tolerated this procedure well and were active throughout the exposure periods.

Development of a two-hit model of cigarette smoke exposure and environmentally acquired *P. murina* infection. Having developed an environmental method of exposing mice to cigarette smoke for prolonged periods, we sought to determine whether *P. murina* infection would alter the course of smoke-induced lung disease. The experimental plan is depicted schematically in Fig. 1. Mice were first exposed to cigarette smoke or air for 1 month. These two groups of mice then were divided further by challenging half the mice in each group with exposure to a *P. murina*-infected SCID mouse for a period of 7 days. At monthly intervals, *P. murina*-infected mice were rechallenged by exposure to a *P. murina*-infected

SCID mouse for 3 days. Using real-time PCR, preliminary studies showed that the lungs of all C57BL/6 mice contained detectable *P. murina* after 3 days of cohousing (data not shown). However, we elected to extend the exposure period to 7 days for the initial infection, to ensure that transmission of adequate intensity had occurred. Each group of mice maintained their housing assignment for the duration of the experiments. At 2 and 4 months, groups of mice were euthanized and measurements of *P. murina* infection, pulmonary function, and morphometry were conducted. Based on pilot work to estimate variability in the parameters measured, we performed the experiments using five mice per group. The experiments were performed four times. There were no differences in body weights among the groups of mice during these experiments.

To control for any environmental stress caused by exposure of the C57BL/6 mice to a cohoused SCID mouse, we performed parallel experiments using uninfected SCID mice. In each case, PCR confirmed the absence of *P. murina* rRNA in the lungs of the uninfected SCID mice and in the cohoused C57BL/6 mice. Furthermore, we detected no inflammatory or cellular changes in the lungs of the C57BL/6 mice exposed to uninfected SCID mice, and measurements of their lung physiology and morphometry were not affected by this exposure.

Real-time PCR assay for *P. murina* rRNA. *P. murina* rRNA was measured using a modification of the method of Zheng et al. (55), as previously used by our group (6). The left lung from each mouse was frozen in liquid nitrogen and homogenized in 1 ml UltraSpec (Biotecx) with RNA isolated per the manufacturer's instructions, and 5 μl of the preparation from each mouse was used for PCR. Random hexamers were used to generate cDNA (TaqMan, Applied Biosystems). The forward and reverse PCR primers for the targeted portion of *P. murina* rRNA (GenBank accession number AF257179, a large-subunit rRNA gene) were 5′-ATG AGG TGA AAA GTC GAA AGG G-3′ and 5′-TGA TTG TCT CAG ATG AAA AAC CTC TT-3′, respectively, and the probe was 6-carboxyfluorescein–AAC AGC CCA GAA TAA TGA ATA AAG TTC CTC AAT TGT TAC–6-carboxytetramethylrhodamine. Real-time PCR was performed using TaqMan reagents and an ABI Prism 7000 cycler (both from Applied Biosystems) for 50 cycles (95°C for 20 s and 60°C for 60 s). The copy threshold cycle values were converted to rRNA copy number using a standard curve prepared from a recombinant standard (generously provided by Chad Steele, University of Pittsburgh), and the detection limit was approximately 100 copies. Samples and standards were run in triplicate.

Measurements of pulmonary function. Pulmonary function testing on mice was performed as previously described (51). Briefly, mice were anesthetized by intraperitoneal injections with ketamine and xylazine. A 19-gauge metal tracheostomy tube, fitted with an adaptor to attach to a mechanical ventilator, was inserted into the trachea and was secured with a nylon suture. Mice were placed on a mechanical ventilator in a sealed plethysmograph as part of the Pulmonary Maneuvers System (Buxco Electronics). Measurement of FRC was done by occluding the airway while the anesthetized mouse attempted to breathe spontaneously. The pressure that the mouse exerted and the change in the mouse's thoracic volume were recorded, allowing the calculation of FRC using Boyle's law. A quasistatic pressure/volume maneuver was performed, allowing measurements of lung compliance (slope of the pressure-volume curve between 0 and 10 cm H_2O pressure) and TLC. Data for all maneuvers were analyzed using Biosystem XA software (Buxco Electronics). The total time of mechanical ventilation required for these measurements was less than 10 min.

Measurements of lung morphometry. Mice were euthanized by intraperitoneal injection of pentobarbital sodium and were exsanguinated by aortic transection. Excised lungs were inflated with 10% buffered formalin at 25 cm H_2O pressure and were stored in 10% buffered formalin for 24 h. The left lung from each mouse was removed and sectioned into 3-mm slices along the long axis. A random number was generated to determine which sections were chosen for morphometric analysis. The sections were embedded in paraffin, and then 5-μm sections were cut, fixed to glass slides, and stained with hematoxylin and eosin. Morphometry was performed using Computer-Assisted Stereology Toolbox (Olympus, Denmark) software. Using a 4× low-power objective, the entire area of lung from each tissue section was outlined and included in the sampling area. Under a magnification of ×20, a computer-assisted sampling protocol chose a random starting field from the selected area, and subsequent fields were determined by using a fixed step in the *x* and *y* positions via a computer-controlled microscope stage (Prior). Thus, a random sampling of the entire tissue section was examined, allowing for objective measurements. A computer-generated grid of line segments of known length was superimposed on each field. The number of alveolar intercepts of these lines was counted. These data were used to calculate the mean linear intercept (MLI) based on a modification of the method first described by Dunnill (18), using the following formula: MLI = Σ(length of all line segments)/number of alveolar wall intercepts. In all cases, the evaluator was blinded to the treatment condition of the mice.

TABLE 1. Pulmonary function testing in air- and smoke-exposed mice[a]

Exposure	FRC (ml)	TLC (ml)	Compliance (ml/mmHg)
Air	0.342 ± 0.010	1.028 ± 0.020	0.049 ± 0.001
Smoke	0.395 ± 0.010	1.105 ± 0.018	0.053 ± 0.001
P value	0.003	0.005	0.040

[a] Pulmonary function testing was performed on mice exposed to air alone or to cigarette smoke for 6 months as described in the text. These experiments were performed in the absence of *P. murina* exposure. Means ± SEM are shown (n = 10 mice per group), and the results were compared by *t* test.

Histologic analysis. Inflammatory infiltrates in tissue sections stained with hematoxylin-eosin were evaluated using a scoring system previously described and validated (40). Scoring was performed without knowledge of the treatment group, using a scale of 0 (no inflammation) to 5 (severe perivascular and peribronchial inflammation with effacement of alveolar parenchyma and airways by sheets of inflammatory cells). To evaluate the integrity of elastin fibers in the lung, tissue sections were stained in parallel using a modified Van Gieson stain (Accustain Elastin; Sigma). To perform semiquantitative scoring for intensity of elastin staining, we modified a grading system previously described for rat lungs (4). This scale varies from 0 (no detectable elastin staining) to 5 (elastin staining equivalent to that for an untreated control mouse). Sections were scored without knowledge of treatment group.

Quantification of inflammatory cell phenotypes. For differential counting of lung cells obtained by lavage, mouse lungs were lavaged with 3 ml normal saline and cells were concentrated by centrifugation as previously described (5). Cell pellets were then suspended in phosphate-buffered saline and were counted in a hemocytometer. Lavaged cells were prepared for differential counting by gravity filtration onto nitrocellulose and staining with Carazzi's hematoxylin-eosin, and blinded differential counts were performed on at least 300 cells per slide. To identify cell phenotypes, lung portions were homogenized by mincing with a scalpel followed by digestion in collagenase and DNase. The resultant cell suspension was centrifuged and then suspended in RPMI. After extensive washing of cells to remove debris, flow cytometry was performed as previously described (5). Briefly, aliquots were stained with monoclonal antibodies (Biosciences), conjugated either to fluorescein or to phycoerythrin, at previously defined optimal dilutions in 96-well, round-bottom plates for 30 min. The following lymphocyte markers (and antibody clones) were examined: CD45 (30-F11), CD4 (RM4-5), and CD8 (53-6.7). In each case, duplicate aliquots of cells were examined after staining with irrelevant, isotype-matched controls to excluded nonspecific antibody binding. Flow cytometry was performed using a FACScan from Becton-Dickinson Immunocytometry Systems, Mountain View, CA. Flow cytometry data are expressed as percentages of lymphocytes bearing specific markers, having gated on the general lymphocyte population using forward and right angle scatter.

Statistical analysis. All data are presented as means ± standard errors of the means (SEM) and results compared using the statistical software program Prism4 (GraphPad Software). Group means were compared using Student's *t* test for two-group comparisons. For parametric data with multiple groups, analysis of variance (ANOVA) with Newman-Keuls follow-up testing was performed. For nonparametric data, multiple groups were compared using the Kruskal-Wallis test and Dunn's multiple-comparison follow-up testing. Each group was compared with all others, and significant differences are indicated in the figures. Comparisons with a P value of <0.05 were deemed statistically significant.

RESULTS

Prolonged smoke exposure in mice leads to airspace enlargement. Chronic exposure of mice to cigarette smoke has become the accepted experimental paradigm to model development of emphysema (21, 46). We adapted this method to an environmental smoking chamber as described in Materials and Methods. Mice exposed to cigarette smoke for 6 months were evaluated for changes in pulmonary function and were assessed for airspace enlargement. Table 1 shows the results of

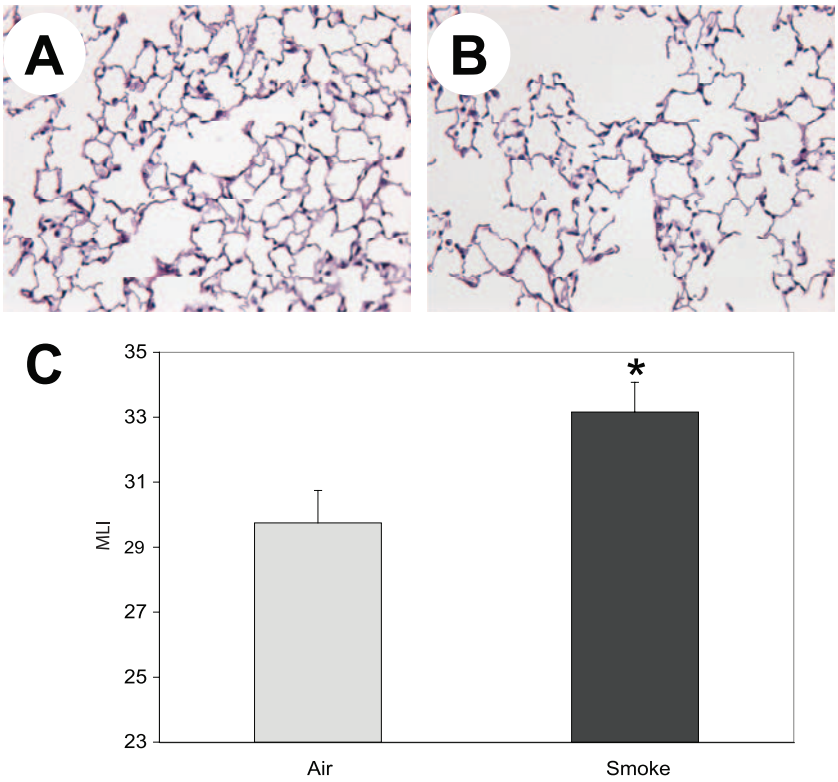

FIG. 2. Prolonged smoke exposure in mice results in airspace enlargement. Mice were exposed to diluted cigarette smoke for 4 h each day, 5 days per week, for 6 months. Mice were euthanized after assessment of pulmonary function, and the lungs were processed for measurement of MLI as described in Materials and Methods. (A and B) Representative sections of lung in air-and smoke-exposed mice, respectively (original magnification, ×10). (C) Results of morphometry measurements. There was increased airspace enlargement (higher MLI) in smoke-exposed mice ($n = 10$ mice per group; *, $P < 0.05$ compared to air-exposed controls by t test). Error bars indicate SEM.

pulmonary function testing. Mice that were exposed to cigarette smoke had significantly increased FRCs, TLCs, and lung compliances compared to mice exposed to air. Airspace enlargement was assessed in these same mice by lung morphometry. Figure 2 shows representative histology of lungs from smoke-exposed and air-exposed mice. Quantitative assessment of airspace enlargement in these mice demonstrated statistically significant increases in MLI in the smoke-exposed group (Fig. 2C). Importantly, data from earlier time points (either 2 or 4 months) did not demonstrate statistically significant changes in MLI compared with air-exposed mice (data not shown). Histologic examination of lung tissue obtained from smoke-exposed mice showed minimal inflammation at the 2-, 4-, or 6-month time point (data not shown). Thus, this model system of prolonged, low-intensity cigarette smoke exposure in environmental chambers results in changes in lung physiology and in airspace enlargement but in relatively little pulmonary inflammation. Additionally, we did not observe significant morphological or physiologic changes until 6 months of exposure had elapsed.

Cigarette smoke exposure increases organism burden 2 months after exposure to *P. murina*. Groups of C57BL/6 mice were exposed to air or to cigarette smoke for a month as described in Materials and Methods. Subgroups of mice were then exposed to a *P. murina*-infected SCID mouse, and either air or smoke exposure was continued for an additional 4 months. At 2 and 4 months after the initial environmental exposure to *P. murina*, groups of mice were evaluated for pulmonary burden of infection. Using a sensitive PCR assay, we found that *P. murina* was present in lungs of smoke-exposed mice at a burden nearly 3 log units higher than that in mice that were not exposed to cigarette smoke (Fig. 3). At 2 months, the lungs of mice exposed to cigarette smoke plus *P. murina* contained significantly higher copy numbers of *P. murina* than the lungs of mice exposed to air plus *P. murina*; this difference did not persist at the 4-month time point. For comparison, the lungs of SCID mice used as sources of infection in these experiments contained copy numbers of 10^9 to 10^{10} per lung. Light microscopic examination of silver-stained lung sections from the environmentally exposed mice demonstrated only rare organisms, but the lungs of the source (SCID) mice contained abundant organisms. As expected, there was no detectable *P. murina* in either smoke-exposed or air-exposed mice that were not exposed to a *P. murina*-infected SCID mouse. Similarly, no *P. murina* was detected in either smoke-exposed or air-exposed mice that were exposed to an uninfected SCID mouse. These data indicate that cigarette smoke significantly increases the burden of *P. murina* in the lung after 2 months of exposure.

***P. murina* infection and cigarette smoke exposure increase TLC.** Pulmonary function testing was performed in groups of mice at 2 and 4 months after the initial exposure to *P. murina*, and the results were compared with data from groups of mice not exposed to the pathogen. At the 2- and 4-month time

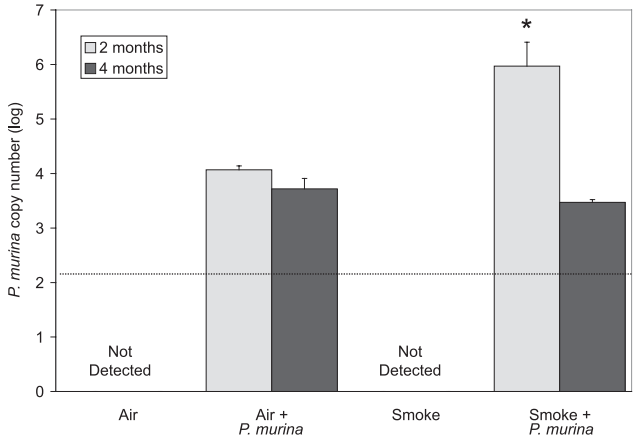

FIG. 3. Smoke-exposed mice have an increased organism burden 2 months after exposure to *P. murina.* Mice were exposed to cigarette smoke or air for 1 month, and then subgroups were exposed to *P. murina* by cohousing with an infected SCID mouse as described in Materials and Methods. Both air- and smoke-exposed cohorts had easily detectable *P. murina* infection after cohousing, but there were significantly higher copy numbers in the smoke-exposed cohort at the 2-month time point. *P. murina* RNA was not detected in the groups that were not exposed to an infected mouse. The dashed line denotes the limit of detection in this assay. For comparison, SCID mice used in cohousing had copy numbers of $\geq 10^9$ (not shown). Data are expressed as mean copy number ± SEM on a log scale ($n = 5$ mice/group; *, $P < 0.05$ compared to other groups with measurable copy numbers by ANOVA).

points, FRC was significantly increased in mice exposed to air plus *P. murina* and in mice exposed to smoke plus *P. murina*, compared to mice exposed to air alone (Fig. 4, top panel). The FRC values for mice exposed to smoke alone did not differ from those obtained for mice exposed to air alone, nor did the FRC values for mice exposed to air plus *P. murina* differ from those obtained for mice exposed to smoke plus *P. murina*. In contrast, when examining TLC, there were significant differences between the 2- and 4-month time points (Fig. 4, bottom panel). At the 2-month time point, mice exposed to air plus *P. murina* or to smoke plus *P. murina* demonstrated significantly higher TLCs than mice exposed to air alone. The values for the mice exposed to air plus *P. murina* were no longer significantly different from those for the mice exposed to air alone at the 4-month time point. In contrast, a significant increase in TLC persisted in the mice exposed to smoke plus *P. murina* compared with mice exposed to air alone. Values from mice exposed to smoke plus *P. murina* were significantly higher than values from mice exposed to air plus *P. murina* at the 4-month time point but were not statistically different than values from mice exposed to smoke alone. While the effect of smoke alone was not significant at 2 months, smoke produced a significant increase in TLC at the 4-month time point compared with mice exposed to air alone. Importantly, the increases after only 2 months of *P. murina* infection in FRC (24% in air-exposed mice and 15% in smoke-exposed mice) and in TLC (11% in air exposed mice and 10% in smoke-exposed mice) are proportionally greater than the increases measured after 6 months of smoke exposure alone (Table 1) (15% increase in FRC and 8% increase in TLC).

***P. murina* and cigarette smoke exposure cause airspace enlargement.** Morphometry measurements were performed in groups of mice at 2 and 4 months after initial exposure to *P. murina* and were compared with data from groups of mice not exposed to the pathogen. After 2 months, there were no significant differences in airspace size among the groups (Fig. 5). After 4 months, however, the values from mice exposed to smoke plus *P. murina* were significantly greater than those obtained from mice exposed to air alone. Values from mice exposed to air plus *P. murina* or to smoke alone did not differ from values from mice exposed to air alone or from values from mice exposed to smoke plus *P. murina*. Within treatment groups, only the group exposed to smoke plus *P. murina* demonstrated significant differences in MLI between the 2- and 4-month time points. As expected, there was no effect of smoking alone after only 4 months of exposure.

To confirm that the airspace enlargement that we measured represented destruction of alveolar walls, we prepared duplicate sections of lung for analysis by elastin staining. We found that the elastin staining results closely paralleled the morphometry data. Semiquantitative scoring of these lung sections, performed without knowledge of treatment groups, confirmed a significant decrease in intensity of elastin staining in the mice exposed to cigarette smoke plus *P. murina*. At the 2-month time point, elastin scores were median grade 5 (normal) in the groups exposed to air alone, smoke alone, and air plus *P. murina* and were 3 in the group exposed to smoke plus *P. murina* ($P < 0.01$ by the Kruskal-Wallis test).

***P. murina* and cigarette smoke exposure interact to cause pulmonary inflammation.** Analysis of histology demonstrated mild inflammation only in mice exposed to both cigarette smoke and *P. murina* (Fig. 6D). Histologic grading of tissue sections showed no significant inflammation in mice exposed to air alone (Fig. 6A), air plus *P. murina* (Fig. 6B), and smoke alone (Fig. 6C) at this 2-month time point. Semiquantitative grading of the inflammatory infiltrates showed a significant increase in inflammation only in mice exposed to cigarette smoke plus *P. murina* (median grade 1 versus median grade 0 for other groups; $P < 0.01$ by the Kruskal-Wallis test).

The phenotypes of cells in bronchoalveolar lavage fluids were enumerated by differential counting. Only the lavage fluids from mice exposed to both cigarette smoke and *P. murina* demonstrated significant increases in numbers of macrophages and lymphocytes compared with the other groups (Fig. 7). Although there was also a trend toward increased numbers of neutrophils in the group exposed to smoke plus *P. murina* compared with the other groups, it was not statistically significant. Because lavage fluid from individual mice did not contain sufficient numbers of lymphocytes to perform phenotyping by flow cytometry, we prepared lung homogenates from individual mice. The lungs of mice exposed to *P. murina* contained significantly greater percentages of $CD4^+$ and $CD8^+$ lymphocytes than the lungs of mice unexposed to *P. murina* (Fig. 8). Additionally, homogenates from mice exposed to *P. murina* plus smoke contained significantly greater percentages of $CD8^+$ lymphocytes than homogenates from mice exposed to *P. murina* alone. Cigarette smoke exposure alone, at the 2-month time point, did not significantly alter the percentages of $CD4^+$ or $CD8^+$ lymphocytes in lung homogenates compared with the air-alone group.

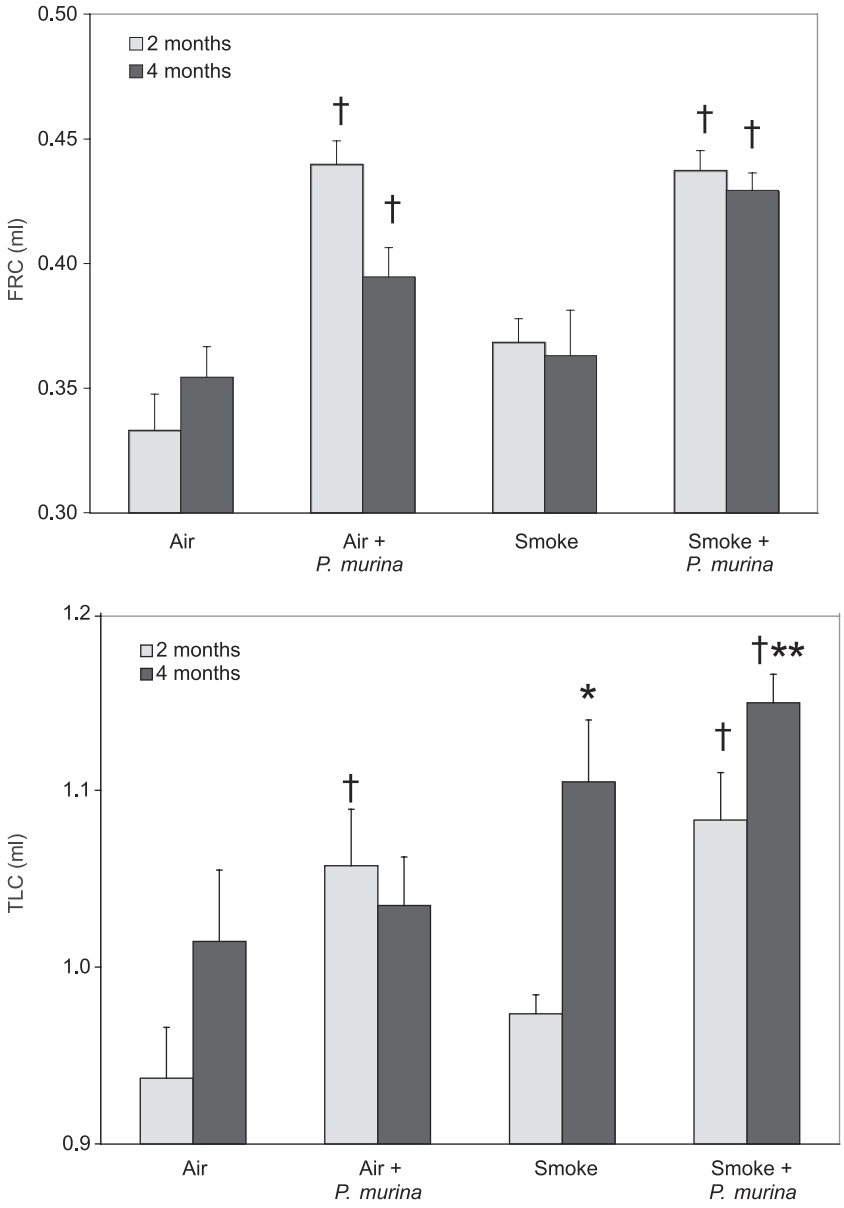

FIG. 4. FRC and TLC are increased 2 months after exposure to *P. murina*, and after 4 months only smoke plus *P. murina* results in increased TLC. Mice were exposed to cigarette smoke or air for 1 month, and then subgroups were exposed to *P. murina* by cohousing with an infected SCID mouse as described in Materials and Methods. After 2 months, FRC (top panel) and TLC (bottom panel) were measured using a whole-body plethysmograph. The data are presented as means ± SEM ($n = 5$ mice/group; *, $P < 0.05$ compared to 2-month data from same condition; †, $P < 0.05$ compared with air alone at same time point, **, $P < 0.05$ compared with air plus *P. murina* at same time point [in all cases by ANOVA]).

DISCUSSION

We report the first data from an animal model examining the in vivo interaction of cigarette smoke and *P. murina*, using environmentally acquired transmission designed to mimic the presumed natural route of infection. Our results show that cigarette smoke exposure increased the lung organism burden of environmentally acquired *P. murina* for at least 2 months. *P. murina* infection, regardless of smoke exposure, produced changes in lung physiology 2 months after initial infection. Importantly, however, after 4 months of exposure, significant increases in TLC and in airspace enlargement (as measured by MLI) occurred in mice exposed to cigarette smoke plus *P. murina.* Only those mice exposed to cigarette smoke plus *P. murina* demonstrated increases in lung inflammation. Taken together, these findings imply that cigarette smoke impairs the host defense against *Pneumocystis* species and indicate roles for *P. murina* and cigarette smoke in the experimental induction of altered physiology and airspace enlargement. Hence, this novel model system has important implications for the investigation of human emphysema, both in HIV-infected individuals and in COPD patients.

The presence of *P. jirovecii* organisms in human respiratory secretions was previously considered to define clinical pneumonia, most likely from reactivation of latent infection in im-

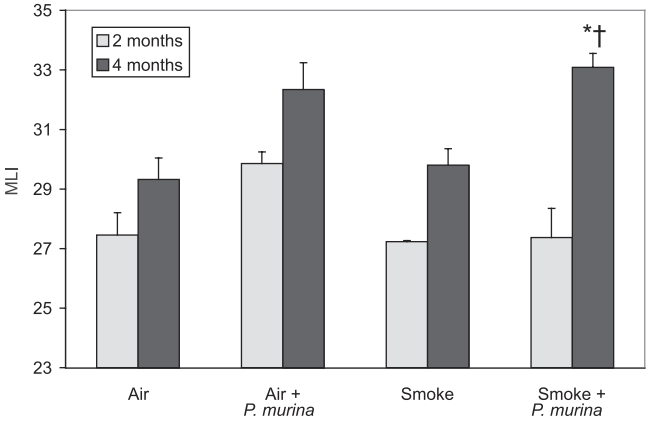

FIG. 5. Airspace enlargement is present in mice after exposure to *P. murina*. Mice were exposed to cigarette smoke or air for 1 month, and then subgroups were exposed to *P. murina* by cohousing with an infected SCID mouse as described in Materials and Methods. After 2 and 4 months, measurement of MLI was performed. There was increased airspace enlargement (higher MLI) in mice exposed to smoke plus *P. murina* compared with air alone at the 4-month time point. While the 2- and 4-month data were not significantly different in other groups, the mice exposed to smoke plus *P. murina* demonstrated a greater MLI at 4 months than at 2 months ($n = 5$ mice/group; *, $P < 0.05$ compared to 2-month data from same condition; †, $P < 0.05$ compared with air alone at same time point [by ANOVA]).

munocompromised hosts. However, more recent data from both human and animal studies have identified carrier states for *Pneumocystis* species, suggesting that transmission and colonization are more likely modes of acquisition (12, 17, 22, 50). Colonization of the respiratory tract with *P. jirovecii*, detected by sensitive molecular methods, occurs in both immunocompetent and immunocompromised individuals. In COPD patients, *P. jirovecii* DNA can be detected at high rates in lung specimens obtained at the time of transplantation or lung resection, compared to lung specimens from patients without COPD (35). Using similar detection methods, *P. jirovecii* is also frequently found in sputum specimens from patients with chronic bronchitis (10). Furthermore, sera from patients with COPD who are colonized with *P. jirovecii* demonstrate increased levels of inflammatory cytokines (interleukin-6, interleukin-8, and tumor necrosis factor alpha) compared with sera from patients with COPD who are not colonized (11). Such results show an association between detection of *P. jirovecii*, inflammation, and COPD.

The finding that cigarette smoke exposure increased the lung organism burden of environmentally acquired *P. murina* in our model has several potential explanations, which are not mutually exclusive. Cigarette smoke could decrease clearance by mechanical means, such as inhibition of mucociliary clearance. It is also likely that immune and inflammatory clearance mechanisms are altered by the exposure to cigarette smoke. Many reports document the effects of smoke exposure on lung

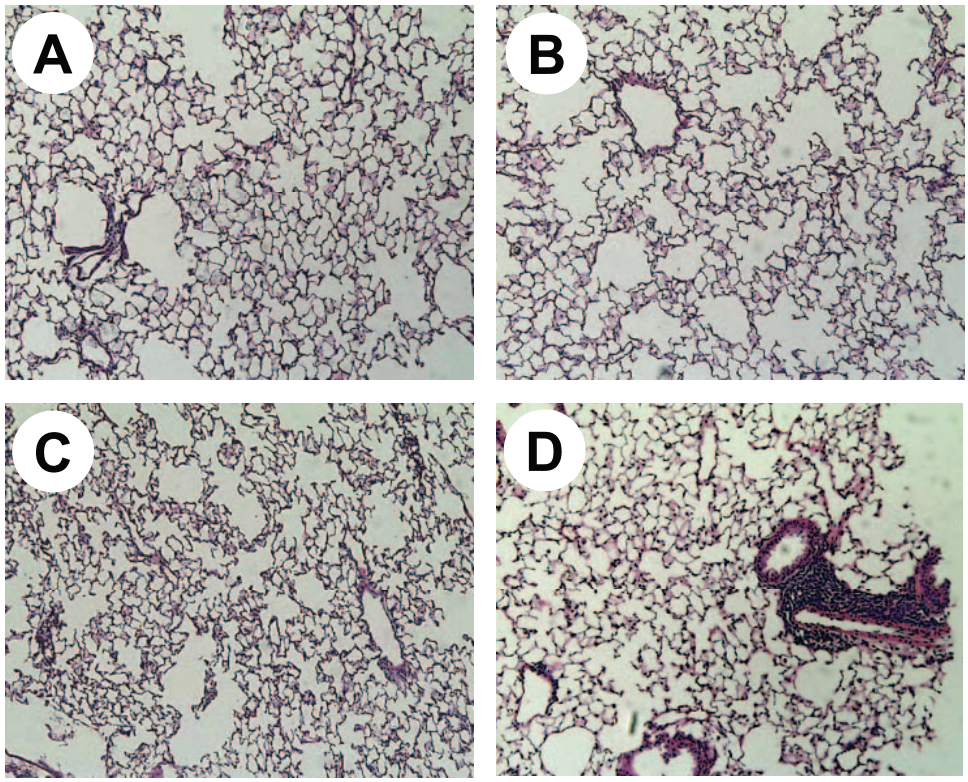

FIG. 6. Airspace enlargement and peribronchiolar inflammation are present in mice 2 months after exposure to *P. murina*. Representative lung sections from mice exposed to air alone (A), air plus *P. murina* (B), cigarette smoke alone (C), and cigarette smoke plus *P. murina* (D) are shown. Significant inflammation in the peribronchiolar areas was seen only in mice exposed to cigarette smoke plus *P. murina*. Hematoxylin-eosin staining was used. Original magnification, ×10.

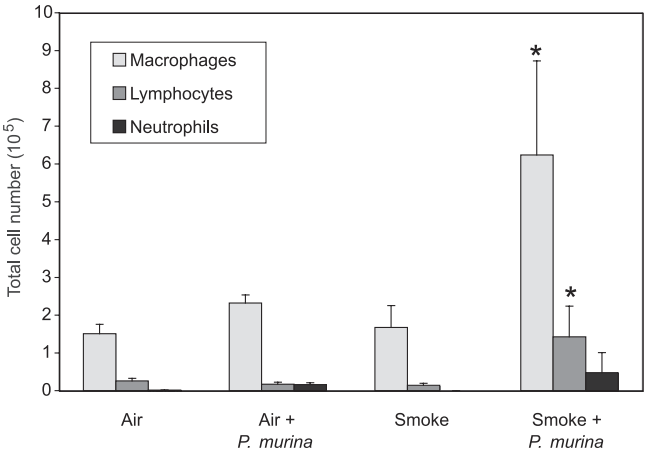

FIG. 7. Enumeration of lung cells in bronchoalveolar lavage fluid demonstrates increases in numbers of macrophages and lymphocytes in mice exposed to cigarette smoke and *P. murina*. Mice underwent bronchoalveolar lavage at the 2-month time point as described in Materials and Methods, and numbers of cells were determined by combining total cell counts with differentials. Only lavage fluids from mice exposed to both cigarette smoke and *P. murina* demonstrated increases in numbers of macrophages and lymphocytes compared with the other groups. The data presented are means ± SEM ($n = 5$ mice/group; *, denotes $P < 0.05$ compared to air alone by ANOVA).

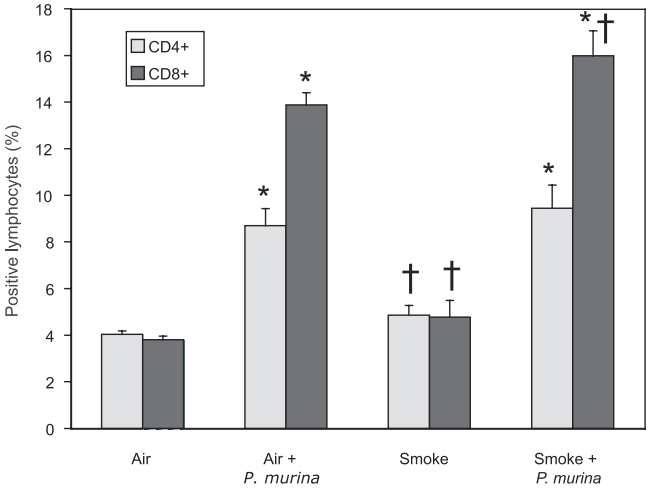

FIG. 8. Phenotyping of lung lymphocytes in homogenates demonstrates increases in percentages of CD4+ and CD8+ lymphocytes in mice exposed to *P. murina*. Lung homogenates were prepared at the 2-month time point as described in Materials and Methods, and phenotypes of lymphocytes (identified by forward- and right-angle scatter and $CD45^+$ staining) were determined by flow cytometry. Homogenates from mice exposed to *P. murina* demonstrated significant increases in percentages of $CD4^+$ and $CD8^+$ lymphocytes. The data presented are means ± SEM ($n = 5$ mice/group; *, $P < 0.05$ compared to air alone; †, $P < 0.05$ compared with air plus *P. murina* [by ANOVA]).

host defense in mice (19, 23, 38). The progression of *P. murina* infection in murine models requires a substantial defect in cell-mediated immunity (24). Mice with intact immune systems do not develop pneumonia but rather develop transient infection (2). *P. murina* infection is evident by PCR at 3 weeks and by microscopic techniques at 4 weeks. Subsequently, an immune response develops, $CD4^+$ and $CD8^+$ T lymphocytes accumulate in the lungs, and *P. murina* is cleared. This entire period of transient infection occurs without overt clinical signs of disease (2). Our results extend this published work by demonstrating an increase in *P. murina* burden in immunocompetent mice during cigarette smoke exposure, indicating a defect in host defense. It is also possible that one or more components of cigarette smoke alter the growth characteristics of *P. murina*. A recent report demonstrates that intraperitoneal infusion of nicotine reduces the intensity of *P. carinii* infection in immunosuppressed rats (49), but significant methodologic differences between that work and the present study preclude direct comparison.

Because smoke exposure increased the burden of *P. murina*, we wondered whether this additional insult would accelerate the physiologic and morphological changes found with prolonged smoke exposure alone. Surprisingly, *P. murina* infection, regardless of smoking status, led to increases in FRC and TLC at 2 months after infection. The magnitude of this change was greater than the changes observed after 6 months of smoke exposure. After 6 months, we measured significant increases in TLC in the mice exposed to cigarette smoke plus *P. murina*. We also observed decreased elastin staining in lung sections obtained from mice exposed to cigarette smoke plus *P. murina*. There are several potential mechanisms for these changes. The initial infection with *P. murina* triggers a primary immune response in the lung. This immune response, either through a direct injury or indirectly via activation of a protease cascade, may have led to the physiologic and morphological changes that we measured. It is also possible that there is a direct effect of the organism itself (e.g., activation of a protease cascade by a *P. murina*-specific protease [1, 3, 26, 41]). These data strongly support a recent report examining primates infected with simian immunodeficiency virus after prolonged colonization with *P. macaca* (36). These primates developed progressive deterioration in their pulmonary function compared with primates not colonized with *P. macaca*, suggesting that prolonged *P. macaca* colonization can result in lung damage.

Based on published work from our laboratory and others, we suspected that modulation of T lymphocytes could underlie the changes in organism burden, morphology, and physiology that we observed. Previous work has demonstrated that $CD4^+$ T lymphocytes are key host defense cells controlling the development of *P. murina* pneumonia in mice (20, 47). However, $CD4^+$ T lymphocytes cause fatal hyperinflammatory reactions after reconstitution of SCID mice in some models (39). Similarly, $CD8^+$ T lymphocytes have been shown to partially protect against *P. murina* pneumonia (5) but also to drive alterations in lung compliance and inflammation (53, 54). In humans, it is tempting to speculate that chronic or repeated infections with *P. jirovecii* may also contribute to the development of accelerated emphysema in individuals with HIV infection. The development of emphysema correlates with increased numbers of $CD8^+$ cytotoxic T lymphocytes in the lungs of susceptible individuals (15, 16). Interestingly, we found that *P. murina* exposure increased the percentages of $CD4^+$ and $CD8^+$ lymphocytes in lung homogenates at the 2-month time point, while cigarette smoke exposure alone did not alter lymphocyte phenotypes. In mice exposed to cigarette smoke plus *P. murina*, percentages of $CD8^+$ lymphocytes were increased

compared with those in mice exposed to air plus *P. murina* or to smoke alone. Our data support recent work demonstrating that $CD8^+$ T lymphocytes are required for the development of cigarette smoke-induced emphysema in mice, mediated through production of gamma interferon-inducible protein-10 (28). It is possible that the mechanism underlying the interaction of cigarette smoke and *P. murina* in driving development of emphysema begins with *P. murina*-mediated $CD8^+$ lymphocyte recruitment to the lung, followed by activation by cigarette smoke. Taken together, these findings advance recent evidence in humans and animals indicating that inflammatory events mediated through $CD8^+$ lymphocytes are important in the pathogenesis of emphysema (52).

Our methods of cigarette smoke exposure and *P. murina* transmission are key elements of experimental design that merit discussion. By inducing physiologic and morphological changes using a protocol that provides less intense, but more prolonged, daily exposure, our results confirm and extend previous murine studies (21, 46). Using a short-daily-duration, high-intensity smoke exposure, Hautamaki et al. noted airspace enlargement compatible with emphysema but not until 6 months of exposure (21). Our methods provide significant advantages over the previously described methods, in which mice are restrained during smoke exposure. Additionally, our methods reduce stress to the mice and permit increases in the sizes of experiments. Another important feature of this model is the use of environmental exposure to *P. murina*, which occurred by cohousing of immunocompetent mice with an infected SCID mouse. We chose this method to model what might occur in humans who have contact with individuals infected with, or colonized by, *P. jirovecii*. Although this method cannot guarantee that all exposed mice receive exactly the same exposure to organisms, our PCR measurements of *P. murina* burden in the source SCID mice were comparable across groups. Importantly, we did not detect physiologic, morphological, or inflammatory changes in mice exposed to uninfected SCID mice. Thus, the possible confounding influence of environmental stress caused by exposure to an unfamiliar mouse cannot be causing the changes we measured.

Finally, these results support the concept that murine models involving cigarette smoke alone fail to induce lung disease closely resembling human COPD because an additional "hit" beyond cigarette smoke is required. Irritants, such as acrolein, have been used to induce emphysematous changes and to demonstrate that $CD8^+$ lymphocytes contribute to macrophage accumulation and the development of airspace enlargement (8). Other investigators have explored the participation of infectious pathogens in the development of emphysema, including latent adenovirus infection (31), chronic *Chlamydia pneumoniae* infection (9, 25), and *Haemophilus influenzae* infection (34). Challenge with cigarette smoke plus lipopolysaccharide has been shown to modulate gene expression in the lung, but physiologic changes were not assessed (30). We chose to study infection with *P. murina* in our model. In humans, the relationship between cigarette smoking and *P. jirovecii* pneumonia remains controversial. While some investigations identify a mild protective effect of cigarette smoking on development of *P. jirovecii* pneumonia in HIV-infected individuals that just achieves statistical significance (42), other investigations have failed to show a statistically significant association (13). More recent work has identified a doubling or tripling of risk of *P. jirovecii* pneumonia in HIV-infected patients who smoke, even after controlling for CD4 count, HIV load, and use of antiretroviral medications (32, 33).

In summary, we have developed a clinically and pathologically relevant animal model to examine the interaction between cigarette smoke and *P. murina*. In this model, significant changes in organism burden occurred after 2 months of *P. murina* exposure, and lung physiology and lung morphology changes were present after 4 months of *P. murina* exposure. In contrast, mice exposed to cigarette smoke alone required 6 months of exposure to develop airspace enlargement. Mice exposed to both cigarette smoke and *P. murina* developed pulmonary inflammation, characterized by accumulation of macrophages and lymphocytes. These findings raise provocative questions about the role of *Pneumocystis* species in the development of pulmonary emphysema and the role of cigarette smoke exposure in the development of pulmonary immunity. Further studies to explore these results in this novel animal model, including study of immunodeficient mice, and to corroborate these findings in human emphysema are warranted.

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